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14. ABSTRACT

Homologous recombination is a key cellular pathway to repair or tolerate complex DNA damage such as DNA double-stranded breaks, interstrand DNA crosslinks, or single-stranded DNA gaps. In addition, homologous recombination is required for the recovery of stalled or broken replication forks. The significance of these functions is highlighted by the use of physical (ionizing radiation) and chemical (topoisomerases inhibitors, interstrand crosslinkers) agents as principal modalities in anti-tumor therapy. The importance of the homologous recombination pathway for breast cancer is underlined by the critical function of the breast cancer tumor suppressor protein BRCA2 in RAD51 filament assembly, a central step in recombination. The objective of the research is to isolate small molecule inhibitors of homologous recombination to allow selective ablation of this pathway. Specifically, we are performing high throughput screens for two target proteins: the double-stranded DNA motor protein RAD54 and the DNA structure-selective endonuclease MUS81-EME1. We have adapted a high throughput ATPase assay for RAD54 and are currently purifying the large amounts of human RAD54 required to perform the screen. For MUS81-EME1, we have designed a fluorescence resonance energy transfer assay and validated as well as quantified MUS81-EME1 cleavage of the fluorescent substrates.

15. SUBJECT TERMS
Homologous recombination, small molecule inhibitor, high-throughput screen

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Introduction:
The project "Exploiting Synthetic Lethal Relationships: Chemical Inhibition of Recombinational Repair as a Strategy to Selectively Target Tumor Cells" aims at isolating small molecule inhibitors of homologous recombination, specifically of the dsDNA motor protein RAD54, a potent ATPase, and of the DNA structure-selective DNA endonuclease MUS81-EME1. The purpose to isolate inhibitors of recombinational repair is to sensitize tumor cells towards therapeutic modalities that are based on DNA damage that is normally repaired by homologous recombination, such as interstrand crosslinks, one- and two-ended double-strand breaks, and single-stranded gaps.

Body:
This report covers the first year of this project and is structured according to the original Statement of Work.

Abbreviations:

FRET:	Fluorescence Resonance Energy Transfer
HTS:	High Throughput Screen
SMDC:	Small Molecule Discovery Center
UCSF	University of California, San Francisco

UC Davis

Task 1 (Months 1-3):	*In vitro* mutagenesis.
Deliverables:	Mutant genes *RAD51-K133R, RAD54-K189R and* MUS81-D307A in *E. coli*

The *RAD51-K131R* mutant was kindly provided by Dr. K. Knight (University of Massachusetts Medical School) as a clone in pET15b, a bacterial expression vector used for RAD51 protein purification. The RAD54 mutagenesis is in progress. A new graduate student, Sucheta Mukherjee, from the Pharmacology and Toxicology Graduate Group will be joining my laboratory and this project. She will focus on MUS81-EME1 and expect quick progress on making the necessary MUS81-EME1 clones and mutants in the first 3 months of year 2.

Task 2 (Months 1-36):	Protein purification for assay development, HTS, secondary assays and screens
Deliverables:	Purified proteins: RAD51, RAD51-K133R, RAD54, RAD54-K189R, MUS81-EME1, MUS81-D307A-EME1

We have purified sufficient quantities of human RAD51 protein for the entire project, which is active in ATPase and *in vitro* recombination assays as previously published (*1-4*). The purification of human RAD54 from baculovirus infected insect cells was established in the laboratory, and the resulting protein is as active in ATPase and *in vitro* recombination as previously published (*5, 6*). Currently, we are in the late stages of a large scale preparation to have sufficient human RAD54 purified for the HTS. The mutant proteins are required later in the re-screening/verification stages of the HTS and will be purfied in year 2. Purification of MUS81-EME1 wild type and mutant proteins

was planned for year 2 and, with Sucheta Mukherjee joining the project, we are on track to achieve this goal.

Task 3 (Months 3-6): Development of malachite green ATPase assay for RAD51/RAD54
Deliverable: HTS assay for RAD51/RAD54

Dr. Kirk Ehmsen, the postdoctoral fellow working on this project, has succeeded to develop and optimize the malachite green ATPase assay (7) for human RAD54. Assay parameters were established in the 384-well format with two goals: (1) to maintain ATP at non-limiting concentrations up to 20 minutes reaction time, at which point the malachite green reagent is added to stop the reaction and begin color development as a function of phosphate quantity, and (2) to optimize a Z' score between 0.5 and 1.0 to validate that the detection window is sufficiently large and that variation between replicates is small. Specifically, the assay was empirically optimized for ATP, RAD54, $Mg(OAc)_2$, and DNA concentration, as well as for components of the malachite green phosphate detection reagent. Figure 1 shows an example of phosphate production dependent on RAD54 concentration, for both the *S. cerevisiae* and human RAD54 proteins. Furthermore, the assay is robust in the presence of DMSO, the primary solvent in which many of the compound library molecules is delivered. Finally, the assay Z' score was optimized to 0.79, indicating that the assay is of sufficient sensitivity and reproducibility to proceed to high-throughput applications.

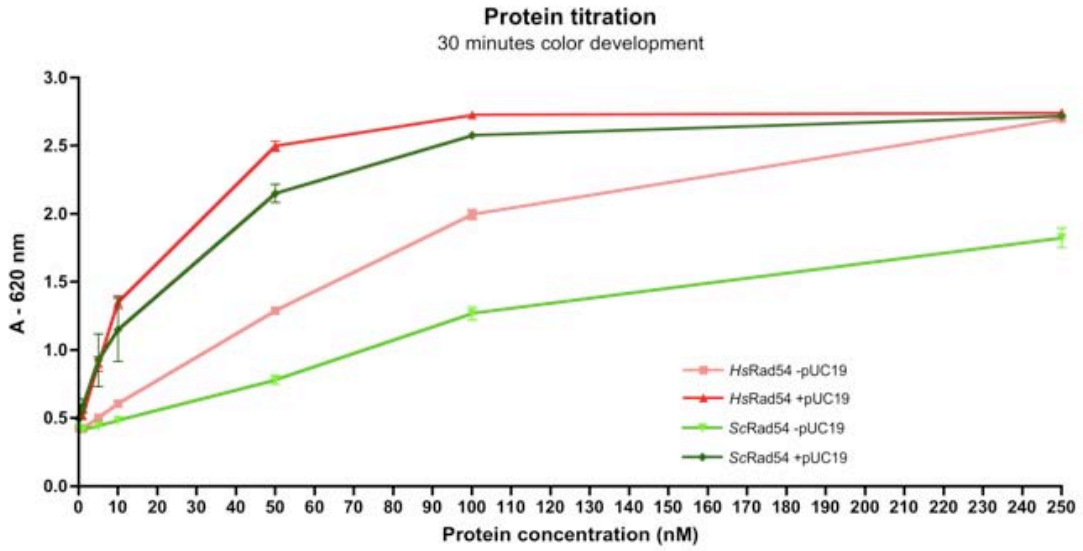

Figure 1. Malachite green-based HTS ATPase assay.
The malachite green assay reports phosphate production dependent on RAD54 concentration, defining a sensitivity window for the identification of RAD54 ATPase inhibitors.

Task 4 (Months 6-9): Pilot screen of LOPAC1280 with ATPase assay for Rad51/Rad54
Outcome: Robust assay for HTS, possible lead candidates

In consultation with Dr. Arkin, we have decided against the originally planned pilot screen by hand-pipetting in our laboratory. Instead we will perform a pilot screen in the SMDC at UCSF.

Task 5 (Months 6-12): Development of FRET-based nuclease assay for MUS81-EME1
Deliverable: HTS assay for MUS81-EME1

A new graduate student, Sucheta Mukherjee, from the Pharmacology and Toxicology Graduate Group spent her rotation in my laboratory, making considerable progress on the development of a FRET-based assay for MUS81-EME1. Of particular importance is whether the position of the fluorescence labels in the oligonucleotide substrate interfere with endonuclease activity. Using fluorescent-labeled DNA substrates that were simultaneously labeled radioactively (^{32}P) at one 5'-end and the purified yeast Mus81-Mms4 protein, we showed in kinetic experiments that the fluorescence labels do not interfere with endonuclease activity in comparison to several control substrates (*8, 9*). By determining the kinetic parameters, K_M and k_{cat}, we show that there is no significant difference between substrates with or without fluorescent labels. We have begun to use FRET as the assay readout, and are currently determining, which instrument and plates are most suited.

UCSF
Dr. Arkin
Task 6 (Months 3-36): Consultation for HTS assays

Dr. Arkin provided helpful consultation in the development of the HTS assays for RAD54 (ATPase) and MUS81-EME1 (FRET). This resulted in reassessing the original plan to conduct a pilot screen by hand, and we will now perform a pilot screen by robotics at SMDC (UCSF) to better test the real time conditions of the HTS screen.

Dr. Arkin and postdoctoral fellow
Task 7a (Months 9-12): HTS (8,000 compounds) with ATPase assay for Rad51/Rad54 and troubleshooting
Deliverable: Possible lead candidates

The limitation of having sufficient quantities of human RAD54 protein has delayed the HTS.

Key Research Accomplishments:
• Generated key materials for the project including purified human RAD51 and human RAD54 protein.

• Established and optimized an HTS assay for human RAD54.

• Established the feasibility of a FRET-based assay for the human MUS81-EME1 endonuclease.

Reportable Outcomes:
None so far.

Conclusions:
We have successfully established an HTS assay for human RAD54 protein that is robust and provides a sufficient screening window to isolate small molecule inhibitors. The HTS screen itself has been delayed, because of initial difficulties to produce sufficient amounts of purified human RAD54 from baculovirus-infected insect cells. While there is no fundamental problem, as the expression and purification has been reported before, and in fact achieved before in my laboratory, the size of the culture dictated by the amounts of protein needed provided a challenge. However, with the HTS assay established, we are now fully focused on protein purification, and, in fact, a large-scale preparation is currently underway.

We are aiming to isolate the first known small molecule inhibitors of homologous recombination, a key DNA repair pathway. Such inhibitors will be an invaluable tool for research, and moreover, have significant potential to act sensitizers in DNA damage-based tumor treatment modalities.

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